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14. ABSTRACT

The goal of this project is to evaluate the potential of pesticides and other compounds used by the military for their potential to damage the brain dopamine system and increase the risk for Parkinson's disease. Notable research accomplishments over the past year include the following: Deltamethrin increased the expression of DAT, TH, and VMAT2, locomotor activity in C57BL mice exposed during development. We further identified transcription factors that underlie this altered exposure. We also showed the pyrethroid treated mice exhibit altered response to cocaine and amphetamine. Deltamethrin did not exacerbate the toxicity to the dopamine neurotoxin MPTP either given before or after MPTP. Thus, with all of the compounds studied we have not observed toxicity consistent with a compound that would be thought to cause overt damage to the dopamine system. However, we have seen alterations of the dopamine system that must be studied further. The completion of this study will reveal the impact of militarily relevant agents on the pathogenesis of Parkinson's disease and response to psychostimulants and hopefully lead to strategies and policies that reduce the incidence of the disease.

15. SUBJECT TERMS
pyrethroid, acetylcholinesterase inhibition, deltamethrin, pyridistigmine cDNA microarra

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Introduction

This report summarizes the key findings of Year 3 of this project. The following Statement of Work is excerpted from the original project, but has been modified to include the supplement on JP-8 jet fuel. Dr. Miller has established a collaborative relationship with Dr. Jeff Fischer at the University of Georgia that should benefit this project. Dr. Fischer studied pharmacokinetics of JP8 and related compounds at Wright Patterson Airforce Base for 20 years before moving to UGA. While we only proposed to do intraperitoneal injections for our Statement of Work, Dr. Fischer has agreed to let us harvest brain tissue from animals exposed to JP8 vapor. We think this will add significant value to our studies by allowing us to compare the i.p. exposure to the more relevant inhalation exposure. Dr. Fischer has also suggested that we try some dermal exposures and we are currently exploring this possibility.

Statement of Work

Specific Aim 1. Effects of pyrethroids, acetylcholinesterase inhibitors, and JP-8 jet fuel on dopamine uptake, DAT localization, and MPP^+ induced apoptosis in DAT expressing cells. This aim will test the hypothesis that pyrethroids and acetylcholinesterase inhibitors increase MPP^+-induced apoptosis primarily through acting on dopamine uptake. The following experiments will be performed under this aim:

I) Perform dopamine uptake tests on DAT expressing cells treated with deltamethrin, permethrin, chlorpyrifos, pyridostigmine bromide, and MPP^+. Years 1-2

II) Determine the effects of deltamethrin, permethrin, chlorpyrifos, pyridostigmine bromide, JP-8, and MPP^+ on DAT localization in DAT expressing cells. Year 3-4

III) Perform caspase 3 assays on cells treated with deltamethrin, permethrin, chlorpyrifos, JP-8, and pyridostigmine bromide to determine if they cause apoptosis or exacerbate MPP^+-mediated apoptosis. Year 1-2

Specific Aim 2. Examine the effects of pyrethroids, acetylcholinesterase inhibitors, JP-8, and MPTP on mouse behavior and dopaminergic and cholinergic gene and protein expression. This aim will test the hypothesis that the combination of pyrethroids and acetylcholinesterase inhibitors decreases dopaminergic activity and increases cholinergic activity, resulting in impaired locomotion in C57BL/6 mice. *An important feature of this aim is that we will examine behavior, gene expression, protein expression, and neurotransmitter levels in the same animals.*

Aim 2A. Assess effects of pyrethroids, acetylcholinesterase inhibitors, and their combination on mouse behavior. In addition, JP-8 will also be tested. This aim will test the hypothesis that these compounds decrease locomotion and increase anxiety and aggression.

IV) Perform locomotor activity, open field ambulation, elevated plus maze, and social interaction tests on C57BL mice six days after MPTP treatment. 3 days prior to or 3 days following MPTP treatment, mice will be treated with 9 mg/kg of deltamethrin, chlorpyrifos, neostigmine, or the combination of deltamethrin and chlorpyrifos. Year 1, 2

Aim 2B. Immunochemical and neurochemical analysis of dopaminergic and cholinergic systems following pyrethroids, acetylcholinesterase inhibitors, JP-8, and MPTP. This aim will assess the effects of pyrethroids, acetylcholinesterase inhibitors, and MPTP on cholinergic and dopaminergic protein expression and function.

V) On the same mice in Aim 2A perform immunoblotting for DAT, D1, tyrosine hydroxylase, M1 and M2 receptors, vesicular acetylcholine transporter, and choline acetyltransferase. Year 1.5 to 2.5

VI) Perform HPLC analysis of monoamines on mice from Aim 2A. Year 2

VII) On a separate subset of animals treated with deltamethrin, chlorpyrifos, neostigmine, and MPTP, perform striatal dopamine and choline uptake. Year 3.

Aim 2C. Use custom cDNA microarrays to analyze regional changes in dopaminergic and cholinergic gene expression following pyrethroids, acetylcholinesterase inhibitors, JP-8, and MPTP. VIII) We will perform cDNA microarray analysis on midbrain, basal forebrain, and striatum from mice treated with deltamethrin, chlorpyrifos, or MPTP. Years 2 and 3 will contain most of the actual hybridizations. Year 4 will be focused bioinformatic analysis. Years 2,3,4

Scientific Progress

We have made significant progress in our Statement of Work. The manuscript included in last year's report has now been published in Toxicology and Applied Pharmacology (included in appendix) and we are making progress on identifying the mechanism by which pyrethroids alter dopamine transporter (DAT) expression.

The upregulation of DAT by deltamethrin stands out as the most intriguing observation in these studies. This is because deltamethrin and related pyrethroids are currently used in military and general populations and DAT is so critical to numerous brain functions. In an attempt to elucidate how this is occurring we have initiated a series of studies (Figure 1). Based upon our interest in developmental neurotoxicity, we administered deltamethrin to pregnant dams and showed the same upregulation of DAT that we had seen in the adult mice (Figure 2+3). While the developmental exposure was not included in the original statement of work, the fact that both paradigms increase DAT suggest that a common mechanism underlies the upregulation. What we saw in the offspring was quite informative. In addition to the upregulation of DAT, we also observed increases in the vesicular monoamine transporter (VMAT2) and tyrosine hydroxylase (TH). No changes were seen in the enzyme AADC. DAT, VMAT2, and TH are all under the transcriptional control of Nurr1. If exposure to the pyrethroids increased Nurr1 then this could explain how the pesticide increases its expression. Indeed, Figure 4 shows that Nurr1 is upregulated in the deltamethrin-treated animals.

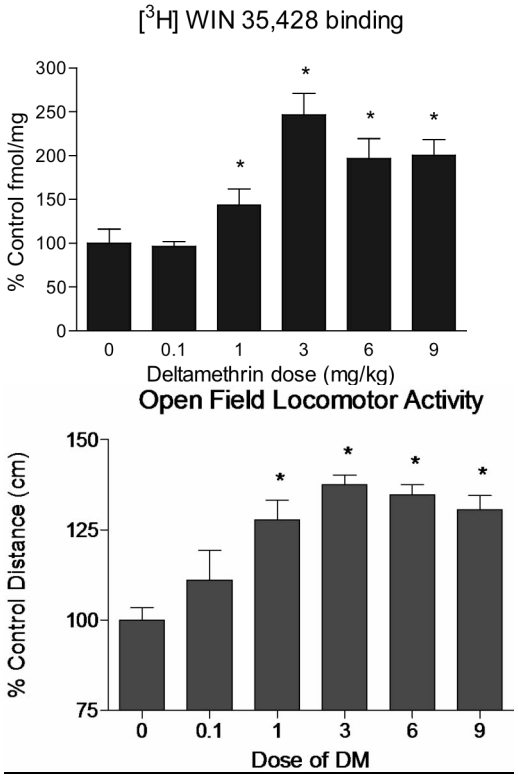

Figure 1. Deltamethrin exposure results in a dose-related increase in DAT levels and locomotor activity**.** C57BL/6j mice were treated with various dosages of DM 3 times over a 2 week period. Locomotor and DAT determinations were made 1 day following the last injection.

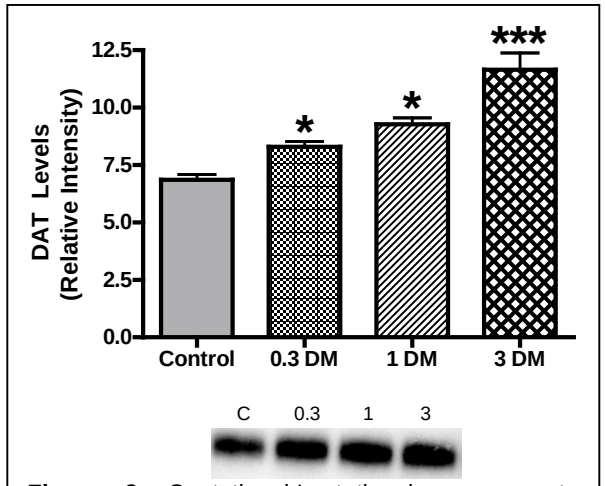

Figure 2. Gestational-Lactational exposure to Deltamethrin (DM) significantly increases DAT levels. Dams were administered corn oil (C), 0.3, 1, or 3 mg/kg DM every 3 days throughout gestation and lactation. Offspring were sacrificed at 5 weeks of age for neurochemical determinations. (Top) Quantification of DAT levels as determined by western blot (Bottom) Representative western blot of DAT from the same animals. N = 4-5 for all experiments. * = $p \leq 0.05$ ** = $p \leq 0.01$ *** $p \leq 0.001$ by Dunnett's test.

As reported in the progress report from the previous year. We have finished the experiments in Specific Aim 1 with the pyrethroids (deltamethrin and permethrin) and cholinesterase inhibitors (chlorpyrifos-oxon and pyridostigmine bromide) and have identified that none of these compounds are capable of directly affecting dopamine transporter (DAT) function in cells stably expressing the human DAT. However, prolonged exposure to the pyrethroid resulted in decreased dopamine uptake. We demonstrated that this

effect was not the result of overt toxicity, but coincided with the appearance of DNA fragmentation, indicative of an ongoing apoptotic process. These results have been published in Toxicology and Applied Pharmacology (*see appendix*). Previously, we provided dose-response data on the effects of JP-8 jet fuel on cytotoxicity in SK-N-MC neuroblastoma cells. We have performed JP-8 exposure studies on mice and are in the process of analyzing that tissue at this point.

In Specific Aim 2, we proposed to examine the effects of pyrethroids, cholinesterase inhibitors, and their combinations on mouse behavior and dopaminergic and cholinergic gene and protein expression. Much of this work, including the effect of pre- or post-exposure to these compounds on MPTP toxicity has been reported previously, but warrants additional discussion. As reported, the single exposures to pyrethroids or cholinesterase inhibitors had no effect on the DAT. We provided preliminary evidence that repeated lower-level exposure to deltamethrin resulted in increased DAT levels. We have extended those observations to include studies with permethrin. We have found that administration of deltamethrin (3 mg/kg) or permethrin (0.8 mg/kg) three times over a two week period to C57BL/6j mice results in a significant increase in DAT-mediated dopamine uptake in striatal synaptosomes prepared from these mice. This up-regulation of DAT function was accompanied by an increase in the number of DAT binding sites as measured by ^{3}H-WIN 35,428 binding to DAT in synaptosomes prepared from these mice. *This is a particularly important finding since military personnel are most likely to be exposed to low-levels of these compounds over a period of time rather than a single large dose.* The dose of deltamethrin administered in these studies (3 mg/kg) is 3-fold lower than the 9 mg/kg used in the single exposure studies reported last year. Likewise, the dose of permethrin (0.8 mg/kg) is 11-fold lower than the 9 mg/kg used in the single exposure studies. Mechanistically, the studies performed in Specific Aim 1 suggest that the effects observed with the *in vivo* exposures are not the result of a direct effect on DAT itself. We have also tested cypermethrin in this paradigm and did not see DAT upregulation with that compound.

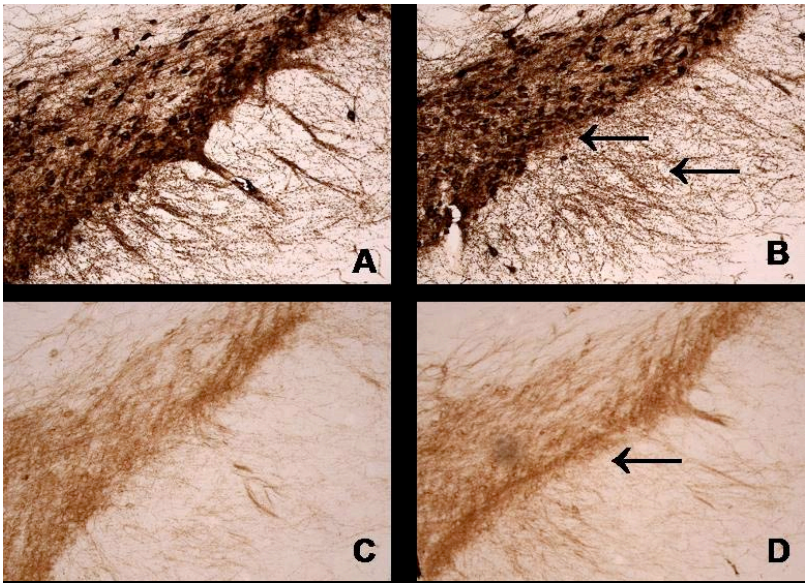

Figure 3. Immunocytochemistry of TH in the SNc offspring of mice exposed to 0 (A) or 3 mg/kg deltamethrin (B) every three days throughout gestation and lactation. Immunocytochemistry of DAT (C=0, D=deltamethrin) in parallel sections of the same mice. Mice were 12 weeks of age at sacrifice.Top arrow points to cell bodies and bottom arrow points to dendritic fibers.

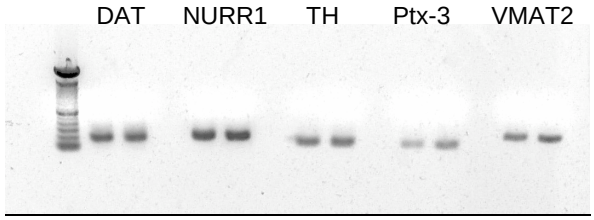

Figure 4. Reverse transcriptase PCR analysis of gene products of the dopaminergic system.

We have extended these studies further with deltamethrin and determined a dose-response relationship between deltamethrin exposure and up-regulation of DAT. As can be seen in Figure 1 (Top), 1 mg/kg administered every three days over 2 weeks appears to be the lowest dosage capable of increases DAT levels. We also determined that the up-regulation of DAT was accompanied by increased locomotor activity in the open-field (Figure 2 Bottom). Indeed, we found a significant correlation between the levels of DAT, as determined by WIN binding, and increased locomotor activity (Figure 3). Therefore, it appears that the functional up-regulation of DAT by deltamethrin exposure has functional consequences on the behavior of the animal that is manifested as hyperactivity. We have also performed preliminary experiments with dopamine receptor antagonists and found that they were able to attenuate the hyperactivity. These data suggest that the hyperactivity is dopaminergic in origin.

Figure 5. Deltamethrin treated mice display increased anxiety as determined by increased time spent in the center of the open field. Top. Control mouse. Bottom. Mouse administered 3 mg/kg deltamethrin.

In addition to the hyperactivity noted in these mice, we have determined that mice exposed to deltamethrin display increased response to cocaine-induced locomotion. We hope to publish these data in the near future. While not part of the original application, these data provide further mechanistic insight into the consequences of low-level pyrethroid exposure. These data also suggest that up-regulation of DAT by pyrethroid exposure may exacerbate responses to therapeutic drugs which work through DAT, such as the anti-depressant and smoking-cessation aid bupropion (Wellbutrin).

In Aim 2, we proposed to determine whether pyrethroid or cholinesterase inhibiting pesticides increased anxiety. Figure 6 demonstrates that mice exposed to 3 mg/kg of deltamethrin every three days for two weeks have decreased anxiety as evidenced by increased time spent in the center of the open-field. Generally, mice fear open space and the center of the open field. The top part of the graph is a track-trace of mouse movement in an open-field for 90 minutes following a 30 minute acclimatization period. As can be seen in the graph, control mice spent the majority of their time in the peripheral part of the open-field box away from the center. However, deltamethrin treated mice were hyperactive and spent more time crossing the open-field and in the center (Figure 5). Whether these results are mainly because of the hyperactivity induced by deltamethrin or truly indicate decreased anxiety remains to be established.

We just completed the following studies and have not had an opportunity to fuly analyze these data. However, we wanted to include these intriguing findings. Permethrin exposure leads to a leftward shift in locomotor response to amphetamine. Since amphetamine causes a reversal of dopamine transport, it could have increased effect on PM exposed mice due to its demonstrated ability to increase the dopamine transporter levels. That is, PM provides more points of exit for intracellular dopamine to be transported to the perisynaptic space after amphetamine exposure.

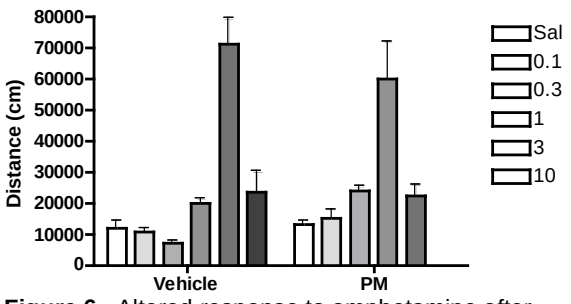

Figure 6. Altered response to amphetamine after permethrin exposure.

Deltamethrin exposed animals do not exhibit a conditioned place preference in response to cocaine (20 mg/kg). This, however, could be due to interference in learning by deltamethrin as opposed to decreasing the reward value of cocaine.

The altered response to psychostimulants is very interesting. This goes beyond the response to drugs like amphetamine and cocaine, and extends to the role of the dopamine system in motivation and reward. We will further explore these findings in the upcoming months.

I anticipate that we will submit 3 manuscripts within the next six months. We have collected a substantial amount of data on the dose-effects of deltamethrin, identified upregulation of transcription factors that may underlie the DAT upregulation, demonstrated heightened response to cocaine, and have completed dosing studies with JP-8. We are also going to obtain brain tissue from our collaborator Jeff Fisher at the University of Georgia, who is conducting pharmacokinetic studies with JP-8 vapor.

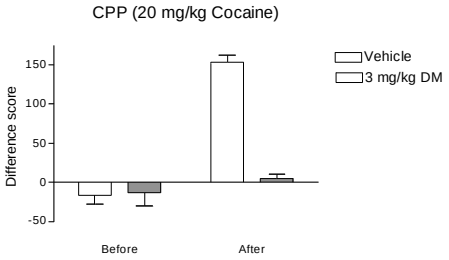

Figure 7. Lack of conditioned place preference to cocaine by deltamethrin.

References

None

Appendix

Copy of manuscript published in Toxicology and Applied Pharmacology

Key Research Accomplishments for Year 4

Identified that developmental exposure causes upregulation of DAT in offspring similar to that seen in adult exposure
Identified transcription factors responsible for upregulation of DAT after pyrethroid exposure
Identified altered response to psychostimulants by pyrethroids

Reportable outcomes:

Poster presented at SOT meeting
Guillot, T.S., Richardson, J.R., and Miller, G.W. (2004). Deltamethrin Increases Dopamine Transporter Expression and Enhances Cocaine-induced Locomotion. Toxicologist 78:1357.

Poster presented at Society of Neuroscience Meeting
Guillot, T.S., Richardson, J.R., and Miller, G.W. (2004). Pesticide Exposure Upregulates the Dopamine Transporter and Increases Cocaine-induced Locomotor Activity while Abolishing Place Preference. Society for Neuroscience Abstracts 804.14.

Abstract submitted for presentation at SOT Meeting
Guillot, T.S., Richardson, J.R., and Miller, G.W. (2005). Sensitive Detection of Behavioral Impairments in Moderately Lesioned MPTP Mice by Automated Gait Analysis. Submitted for SOT Annual Meeting.

Manuscript published

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Pyrethroid pesticide-induced alterations in dopamine transporter function

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Abstract

Parkinson's disease (PD) is a progressive neurodegenerative disease affecting the nigrostriatal dopaminergic pathway. Several epidemiological studies have demonstrated an association between pesticide exposure and the incidence of PD. Studies from our laboratory and others have demonstrated that certain pesticides increase levels of the dopamine transporter (DAT), an integral component of dopaminergic neurotransmission and a gateway for dopaminergic neurotoxins. Here, we report that repeated exposure (3 injections over 2 weeks) of mice to two commonly used pyrethroid pesticides, deltamethrin (3 mg/kg) and permethrin (0.8 mg/kg), increases DAT-mediated dopamine uptake by 31 and 28%, respectively. Using cells stably expressing DAT, we determined that exposure (10 min) to deltamethrin and permethrin (1 nM–100 μM) had no effect on DAT-mediated dopamine uptake. Extending exposures to both pesticides for 30 min (10 μM) or 24 h (1, 5, and 10 μM) resulted in significant decrease in dopamine uptake. This reduction was not the result of competitive inhibition, loss of DAT protein, or cytotoxicity. However, there was an increase in DNA fragmentation, an index of apoptosis, in cells exhibiting reduced uptake at 30 min and 24 h. These data suggest that up-regulation of DAT by in vivo pyrethroid exposure is an indirect effect and that longer-term exposure of cells results in apoptosis. Since DAT can greatly affect the vulnerability of dopamine neurons to neurotoxicants, up-regulation of DAT by deltamethrin and permethrin may increase the susceptibility of dopamine neurons to toxic insult, which may provide insight into the association between pesticide exposure and PD.

Keywords: Deltamethrin; Permethrin; Pyrethroid; Dopamine transporter; Parkinson's disease

Parkinson's disease (PD) is a disabling neurodegenerative disorder characterized by the loss of nigrostriatal dopamine neurons and the formation of intraneuronal inclusions termed Lewy bodies (Olanow and Tatton, 1999). Although the exact etiology of PD is unknown, both genetic and environmental factors are thought to contribute to the pathogenesis of PD. While there are rare instances of genetically-linked PD, data from a recent comprehensive twin study found no significant contribution of genetics to late-onset PD (Tanner et al., 1999). This finding suggests that environmental factors or gene–environment interactions play a pivotal role in the development of sporadic PD.

Several epidemiological studies have identified pesticide exposure as a significant risk factor for Parkinson's disease (Tanner and Langston, 1990; Gorell et al., 1998; Le Couteur et al., 1999; Priyadarshi et al., 2001). Other studies have demonstrated that drinking well-water and living in a rural setting, both of which may increase exposure to agricultural pesticides, increase the risk of developing PD (Rajput et al., 1986; Barbeau et al., 1987; Rajput et al., 1987; Golbe et al., 1990; Semchuk et al., 1991). In addition, exposure to pesticides used in the home has been linked to PD (Stephenson, 2000). However, the majority of studies have not identified specific pesticides or the mechanism by which pesticides damage the dopaminergic system and increase the risk of PD.

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Studies by our laboratory and others have demonstrated that exposure of mice to the organochlorine insecticide heptachlor increases the expression of the plasma membrane dopamine transporter (DAT; Miller et al., 1999a; Kirby et al., 2001) at dosage levels that elicit no overt toxicity. DAT is an integral component of normal dopamine function and is responsible for terminating dopamine neurotransmission by rapid reuptake of dopamine into the presynaptic terminal (Shimada et al., 1991; Giros and Caron, 1993; Miller et al., 1999b). Several studies have demonstrated that alterations in the expression of DAT can greatly affect the vulnerability of the dopamine neuron to neurotoxins such as MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). Gainetdinov and colleagues demonstrated the requirement of DAT for the toxicity of MPTP (Gainetdinov et al., 1997), while Donovan et al. (1999) have shown that overexpression of DAT in transgenic mice results in greater loss of dopamine neurons following MPTP exposure. Therefore, exposure to pesticides that increase DAT may increase the susceptibility of dopamine neurons to endogenous neurotoxic dopamine metabolites or exogenous neurotoxicants by increasing their uptake by DAT.

In addition to heptachlor, exposure of mice to the pyrethroid pesticides deltamethrin and permethrin has been demonstrated to increase DAT-mediated dopamine uptake (Kirby et al., 1999; Karen et al., 2001; Gillette and Bloomquist, 2003). Pesticides in the pyrethroid class are widely used in household and agricultural applications and are popular because of their low mammalian toxicity. Although pyrethroids are often considered environmentally labile, they readily cross the blood–brain barrier and can achieve considerable concentrations in the brain (Anadon et al., 1996). Acute toxicity of pyrethroids is primarily mediated through interaction with sodium channels, leading to prolonged depolarization and hyperexcitation of the nervous system (Narahashi, 1982; Tabarean and Narahashi, 2001). Pyrethroids have also been shown to be potent releasers of neurotransmitters, including dopamine (Eells and Dubocovich, 1988; Kirby et al., 1999). However, the mechanism by which pyrethroids are capable of increasing DAT-mediated dopamine uptake is not clear.

Here, we report that in vivo exposure to deltamethrin and permethrin not only causes functional up-regulation of dopamine uptake, but increased levels of DAT protein as well. In addition, acute exposure of SK-N-MC cells stably expressing DAT to these pyrethroids has no effect on dopamine uptake, indicating that deltamethrin and permethrin do not directly interact with DAT. Finally, we found that longer-term exposure to deltamethrin and permethrin reduces dopamine uptake in these cells, and that this effect is most likely the result of an ongoing apoptotic process. Taken together, our results suggest that the effects of pyrethroids on DAT are indirect and that longer-term exposures may be capable of damaging cells through an apoptotic mechanism.

Materials and methods

Materials. Analytical grade (purity ≥98%) deltamethrin and permethrin were obtained from ChemService Inc. (West Chester, PA). ^{3}H-dopamine (58 Ci/mmol) and ^{3}H-WIN 35,428 (85 Ci/mmol) were purchased from Perkin-Elmer Life Sciences (Boston, MA). The rat monoclonal antibody to DAT was purchased from Chemicon (Temecula, CA) and the monoclonal anti-mouse α-tubulin was purchased from Sigma (St. Louis, MO). The goat anti-rat secondary antibody was purchased from ICN (Costa Mesa, CA) and the goat anti-mouse secondary antibody was from Bio-Rad (Hercules, CA). Super Signal West substrate and stripping buffer were obtained from Pierce (Rockford, IL). Cell culture media and supplements were obtained from Meditech (Herndon, VA). All other reagents were obtained from Sigma or Fisher Scientific (Pittsburgh, PA).

Animals and treatments. Male C57BL/6j mice (8 weeks of age) were obtained from Jackson Laboratories (Bar Harbor, ME). Animals were group housed (6 per cage) under a 12:12 light–dark cycle and acclimatized for 1 week prior to initiation of experiments. Standard rodent chow and tap water was available ad libitum. All procedures were conducted in accordance with the *Guide for Care and Use of Laboratory Animals* (National Institutes of Health) and previously approved by the Institutional Animal Care and Use Committee at Emory University.

A total of 24 mice were used for these experiments. Control mice were injected intraperitoneally with vehicle (methoxytriglycol; $n = 12$) and treated mice were injected with deltamethrin (3 mg/kg; $n = 6$) or permethrin (0.8 mg/kg; $n = 6$) three times over a 2-week period (Days 1, 8, and 15) as described previously (Kirby et al., 1999; Miller et al., 1999b; Gillette and Bloomquist, 2003). One day following the last treatment, striatal tissue was dissected out and prepared for assay as described below.

Synaptosomal dopamine uptake, ^{3}H-WIN 35,428 binding, and Western blot analysis. Dopamine uptake studies were performed as described previously (Miller et al., 1999a). Briefly, crude synaptosomes were prepared from fresh striatal tissue and incubated in assay buffer (4 mM Tris, 6.25 HEPES, 120 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.6 mM ascorbic acid, 5.5 mM glucose, 10 μM pargyline; pH 7.4) containing a saturating concentration of dopamine (1 μM final concentration) and a tracer amount of ^{3}H-dopamine (20 nM). A single saturating concentration of dopamine was chosen to assess effects of pyrethroids on the V_{max} of DAT, since previous studies using the same dosing paradigm have demonstrated no significant effect on K_m (Kirby et al., 1999; Karen et al., 2001). Uptake was allowed to proceed for 3 min at 37 °C, and then terminated by the addition of ice-cold buffer and rapid vacuum filtration over GF/B filter paper using a Brandel harvester. Filters were washed twice more with buffer, allowed to air

dry, and placed in scintillation vials containing 8 ml of Econoscint (Fisher Scientific, Pittsburgh, PA) for scintillation counting. Uptake rates were calculated as specific uptake (total uptake−non-specific uptake), with non-specific uptake defined by the inclusion of 10 μM nomifensine. Following determination of synaptosomal protein concentration (Bradford, 1976), uptake rates were calculated as pmol/min-mg protein and expressed as percentage of control values.

Determination of ^{3}H-WIN 35,428 binding to DAT was performed essentially as described by Coffey and Reith (1994) with modifications to reduce the total volume to 200 μl, for assay in 96-well microtiter plates. Preliminary kinetic studies indicated that the binding of ^{3}H-WIN 35,428 to striatal synaptosomes was best fit to a one-site model determined by non-linear curve fitting techniques (GraphPad Prism 3.0) with a K_d of 6.58 nM and a B_{max} of 1.08 pmol/mg protein. Therefore, binding studies with crude synaptosomes were conducted with a single concentration (10 nM) of ^{3}H-WIN 35,428 in 25 mM sodium phosphate buffer (125 mM NaCl, 5 mM KCl; pH 7.4) for 1 h at 4 °C in 96-well plates. Incubations were terminated by rapid vacuum filtration onto GF/B filter plates and radioactivity was determined by liquid scintillation counting. Non-specific binding was determined by the inclusion of 10 μM nomifensine and specific binding was calculated as the total binding (incubated without 10 μM nomifensine) minus non-specific binding (incubated with nomifensine). Data were calculated as pmol/mg protein and expressed as percentage of control values.

Western blots were performed as previously described (Richardson and Miller, 2004). Briefly, samples (20 μg) were subjected to SDS PAGE on 10% precast NuPage gels (InVitrogen, Carlsbad, CA). Samples were electrophoretically transferred to a polyvinylidene difluoride membrane, and non-specific sites were blocked in 7.5% nonfat dry milk in Tris-buffered saline (135 mM NaCl, 2.5 mM KCl, 50 mM Tris, and 0.1% Tween 20, pH 7.4). Membranes were then incubated in a monoclonal antibody (Chemicon, Temecula, CA) to the N-terminus of DAT (Miller et al., 1997). Antibody binding was detected using a goat anti-rat horseradish peroxidase secondary antibody (ICN, Costa Mesa, CA) and enhanced chemiluminescence. The chemiluminescent signal was captured on an Alpha Innotech Fluorchem 8800 (San Leandro, CA) imaging system and stored as a digital image. Densitometric analysis was performed and calibrated to co-blotted dilutional standards of pooled cells from all control samples. Membranes were then stripped for 15 min at 25 °C with Pierce Stripping Buffer and reprobed with a monoclonal α-tubulin antibody to ensure equal protein loading across samples.

Cell culture. SK-N-MC (human neuroblastoma) cells stably expressing human DAT (SK-DAT; Stephans et al., 2002) were maintained in minimum essential medium (MEM) supplemented with Earle's salts, 10% heat-inactivated fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1 mM non-essential amino acids (Mediatech, Herndon, VA), and incubated under a humidified atmosphere of 5% CO_2 in air at 37 °C. For pyrethroid exposure, deltamethrin and permethrin were dissolved in dimethylsulfoxide (DMSO) at a concentration of 10 mM. Further dilutions were made in uptake buffer for experiments with dopamine uptake and in serum-free media for experiments to determine cytotoxicity and DNA fragmentation. The final concentration of DMSO was ≤0.1% for all experiments and had no effect on any of the parameters studied. Control experiments were performed in the presence of DMSO in a concentration similar to that used in the pyrethroid-treated cells.

^{3}H-Dopamine uptake and Western blot studies in cells. Dopamine uptake by SK-DAT cells was performed as described elsewhere (Pifl et al., 1996). Briefly, cells were plated in 24-well plates and incubated for 48 h in the above MEM medium. Cells were washed once with the uptake buffer (4 mM Tris, 6.25 HEPES, 120 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.6 mM ascorbic acid, 5.5 mM glucose; pH 7.4). For acute studies (10 and 30 min), cells were incubated with various concentrations of either deltamethrin or permethrin (1 nM–10 μM). For longer-term studies (24 h), cells were exposed to the pyrethroids in serum-free media for 24 h and then washed once in uptake buffer. Following the wash step, cells were incubated for 5 min at 37 °C with uptake buffer containing unlabeled DA (2.5 μM) and a tracer amount (20 nM) of ^{3}H-dopamine. Pargyline (10 μM) was included during all the uptake periods to inhibit monoamine oxidase and non-specific uptake was defined in the presence of 10 μM GBR-12935. After the incubation period, the buffer was quickly aspirated off and cells were washed twice with ice-cold buffer. Cells were then dissolved in 0.5 ml of 0.1 M NaOH and the solubilized cellular contents were transferred to liquid scintillation vials containing 8 ml of liquid scintillation cocktail. The radioactivity was measured by scintillation counting and an aliquot of the solubilized cells was used for protein determination using bovine serum albumin as standard (Lowry et al., 1951). Uptake rates were calculated as specific uptake (total uptake−non-specific uptake) and expressed as percentage of control values.

To determine the effects of pyrethroids on the K_m and V_{max} of dopamine uptake in SK-DAT cells, cells were incubated with pyrethroids for 10 min or 24 h and dopamine uptake was determined as described above using increasing concentrations (0.5–40 μM) of dopamine. K_m and V_{max} were determined by non-linear regression using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA).

Western blots were performed as previously described (Miller et al., 1997). Briefly, cells were scraped from culture plates and sonicated at 4 °C in a buffer containing 300 mM sucrose, 10 mM HEPES, and 1 μg/ml of leupeptin,

aprotinin, and pepstatin. Samples (20 μg) were subjected to SDS PAGE on 10% precast NuPage gels (InVitrogen, Carlsbad, CA). Samples were electrophoretically transferred to a polyvinylidene difluoride membrane, and non-specific sites were blocked in 7.5% nonfat dry milk in Tris-buffered saline (135 mM NaCl, 2.5 mM KCl, 50 mM Tris, and 0.1% Tween 20, pH 7.4). Membranes were then incubated in a monoclonal antibody (Chemicon, Temecula, CA) to the N-terminus of DAT (Miller et al., 1997). Antibody binding was detected using a goat anti-rat horseradish peroxidase secondary antibody (ICN, Costa Mesa, CA) and enhanced chemiluminescence. The chemiluminescent signal was captured on an Alpha Innotech Fluorchem 8800 (San Leandro, CA) imaging system and stored as a digital image. Densitometric analysis was performed and calibrated to co-blotted dilutional standards of pooled cells from all control samples. Membranes were then stripped for 15 min at 25 °C with Pierce Stripping Buffer and reprobed with a monoclonal α-tubulin antibody to ensure equal protein loading across samples.

Cytotoxicity and DNA fragmentation assays. The possible cytotoxic effects of pyrethroid exposure on SK-DAT cells was evaluated by measuring lactate dehydrogenase (LDH) leakage into the extracellular fluid using a cytotoxicity detection kit (Roche Applied Science, Indianapolis, IN). Briefly, cells (1×10^4 cells/well) were incubated with different concentrations of pyrethroids (1, 5, or 10 μM) for 24 h in serum-free MEM and the incubation medium was collected and centrifuged. The cell-free supernatant (100 μl) was then mixed with 100 μl of the catalyst-dye mix (included in kit) in a 96-well microtiter plate. LDH activity in the media was determined spectrophotometrically at 490 nm by monitoring the increase in absorbance over a 30-min period. To determine the total amount of LDH in each sample, the original cells and media were lysed in 1% Triton X-100 for 30 min and LDH activity was determined as described above. The LDH release for each sample was defined as the LDH activity in the incubation media divided by the total amount of LDH activity following Triton-lysis and data expressed presented as percentage LDH leakage.

To determine whether pyrethroid exposure resulted in apoptosis in SK-DAT cells, we used the Cell Death Detection ELISA Plus Assay kit (Roche Applied Science, Indianapolis, IN), which provides an index of DNA fragmentation. This kit measures amount of histone-associated low molecular weight DNA, which is indicative of histone-associated DNA fragments which have been cleaved by endonuclease, in the cytoplasm of cells and has been used as a measure of apoptosis in cells exposed to other toxicants (Anantharam et al., 2002; Kitazawa et al., 2002). Briefly, cells were seeded in microplate wells (1×10^4 cells/well) and treated for 24 h in serum-free MEM with either deltamethrin or permethrin. After treatment, cells were pelleted and washed once with phosphate-buffered saline. Cells were then incubated with lysis buffer (supplied with the kit) at room temperature for 30 min and centrifuged. Aliquots of supernatant (20 μl) were dispensed into a streptavidin-coated 96-well microtiter plate (supplied with the kit) and incubated with 80 μl of antibody cocktail for 2 h at room temperature with shaking. The antibody cocktail consisted of a mixture of anti-histone biotin and anti-DNA-HRP, which binds to both single-stranded DNA and double-stranded DNA, which are major constituents of nucleosomes. After incubation, plates were washed with incubation buffer and determination of the amount of nucleosomes retained by anti-DNA-HRP was determined spectrophotometrically with 2,2′-azino-di[3-ethoxybenzyl thiazoline sulfonate] as an HRP substrate (supplied with the kit). Measurements were made at 405 nm using a Spectramax Plus microplate reader (Molecular Devices). Non-specific signal was determined by subtraction of a reagent blank and data were expressed as mU (defined as absorbance $\times 10^{-3}$) cytoplasmic oligonucleosomes.

Statistical analysis. Results were expressed as the mean ± SEM. In instances where data were presented as percentage of control, all statistical procedures were performed on the raw numbers. Data were analyzed by Student's *t* test or one-way analysis of variance (ANOVA). If a significant *F* was determined by ANOVA, post hoc analysis was performed with Dunnett's test. Statistical significance is reported at the $P \leq 0.05$ level.

Results

No overt signs of toxicity, defined as tremor, choreoathetosis, and salivation, were observed following administration of either deltamethrin or permethrin. There were also no significant changes in weight in any of the treated animals (data not shown).

Based upon previous studies demonstrating increased dopamine uptake following deltamethrin or permethrin exposure (Kirby et al., 1999; Gillette and Bloomquist, 2003), we administered deltamethrin (3 mg/kg) or permethrin (0.8 mg/kg) three times over 2 weeks to determine the effects of these compounds on DAT-mediated dopamine uptake and the number of DAT-binding sites. Deltamethrin exposure increased DAT-mediated dopamine uptake in striatal synaptosomes by 31% ($P < 0.01$) 1 day following the last treatment (Fig. 1A). At this same time, permethrin exposure increased dopamine uptake by 28% ($P < 0.01$). The increases in dopamine uptake observed were accompanied by increases in DAT-binding sites as determined by ^{3}H-WIN 35,428 binding in striatal synaptosomes (Fig. 1B). Deltamethrin resulted in a 32% increase ($P < 0.01$), while permethrin exposure increased DAT-binding sites by 24% ($P < 0.01$). The increase in DAT-binding sites and uptake by exposure to deltamethrin and permethrin were accompanied by similar increases (31.2% and 29.3%, $P < 0.01$) in total DAT protein as measured by Western immunoblotting (Fig. 1C).

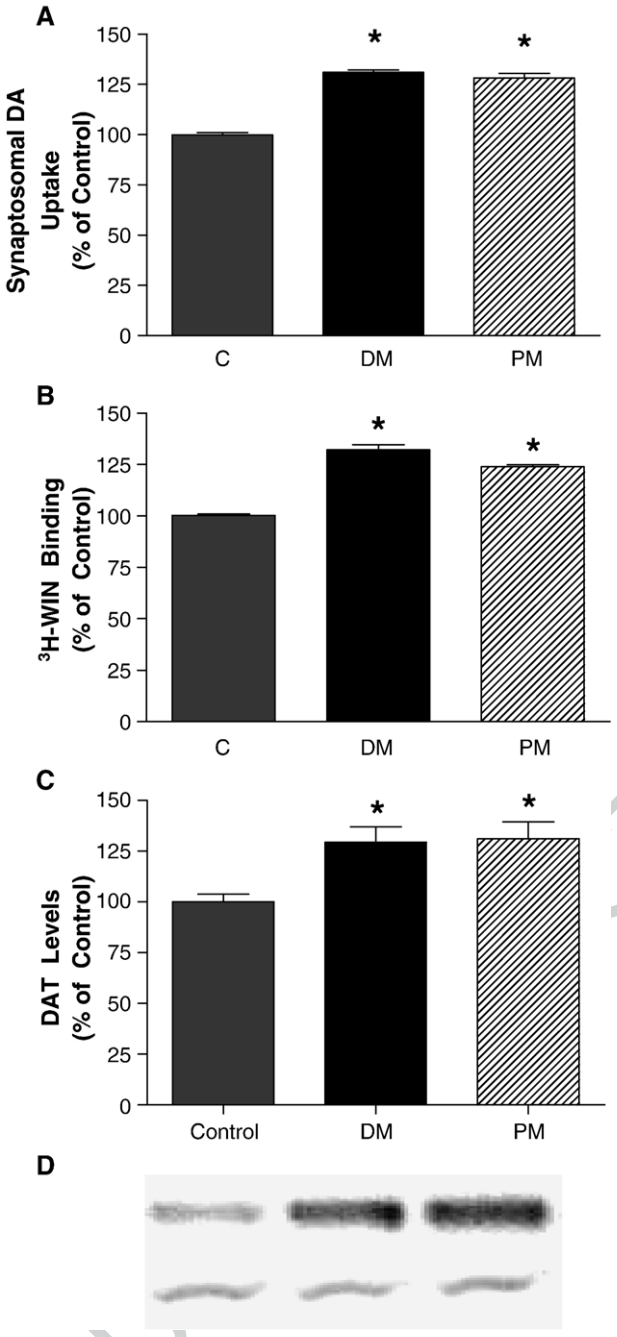

Fig. 1. Repeated administration of deltamethrin (DM; 3 mg/kg) or permethrin (PM; 0.8 mg/kg) to C57 mice increases (A) ^{3}H-dopamine uptake in striatal synaptosomes, (B) DAT levels in striatal synaptosomes as determined by ^{3}H-WIN 35,428 binding, and (C) DAT levels in striatal synaptosomes as determined by Western immunoblotting. Data are presented as percentage of control values and represent mean ± SEM (n = 5–6 animals per treatment for pyrethroids and 12 animals for control). *Groups are significantly different from control values ($P \leq 0.01$) using the untransformed data as determined by ANOVA followed by Dunnett's test. (D) Representative Western blots of DAT (top) and α-tubulin (bottom) from control, deltamethrin-, and permethrin-treated animals.

Since we observed significant up-regulation of DAT following in vivo exposure to deltamethrin and permethrin, we sought to determine whether these effects were the result of direct action of the pyrethroids on DAT. To accomplish this, we exposed SK-N-MC neuroblastoma cells stably expressing DAT (SK-DAT) to various concentrations of pyrethroids for 10 min, 30 min, or 24 h. Exposure of SK-DAT cells for 10 min with either deltamethrin or permethrin (1 μM to 10 μM) had no significant effect on DAT-mediated dopamine uptake (Fig. 2A). Extending the incubation time to 30 min resulted in a significant decrease in dopamine uptake by both deltamethrin (20%; $P < 0.01$) and

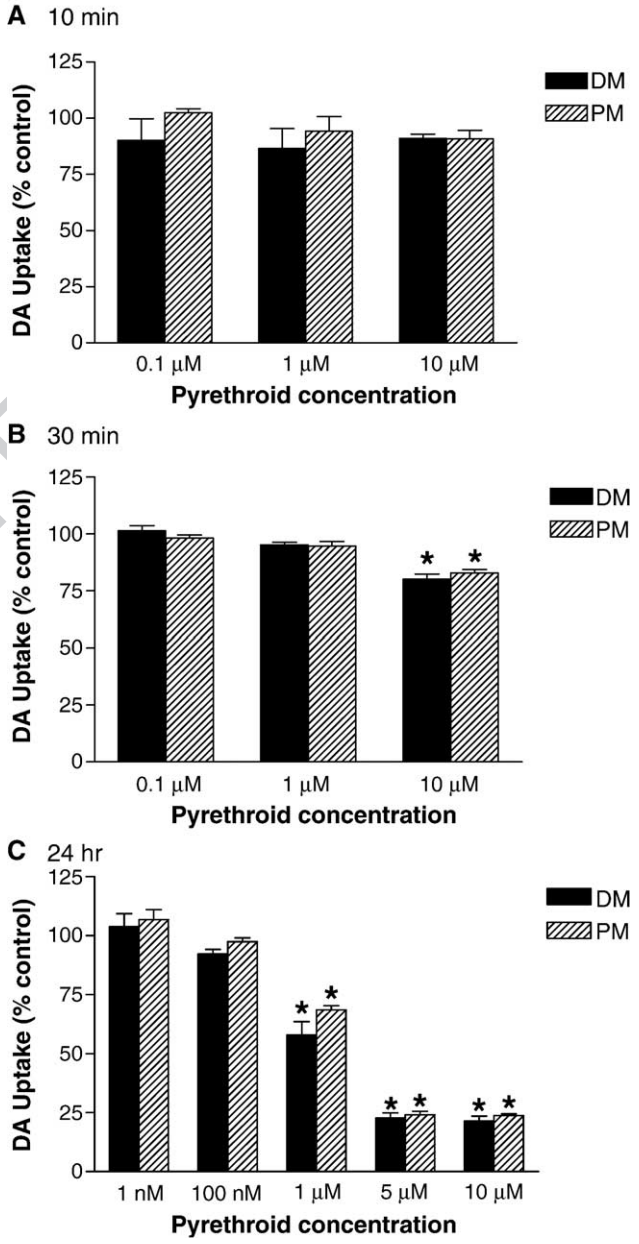

Fig. 2. Effects of deltamethrin (DM) and permethrin (PM) on dopamine uptake in SK-N-MC neuroblastoma cells stably expressing the human DAT. Cells were incubated with various concentrations of DM or PM for 10 min (A), 30 min (B), or 24 h (C) and dopamine uptake was determined as described in Materials and methods. Data are presented as percentage of control values and represent mean ± SEM (n = 3). *Groups are significantly different from control values ($P \leq 0.01$) using the untransformed data as determined by ANOVA followed by Dunnett's test.

permethrin (18%; $P < 0.01$) only at a concentration of 10 μM (Fig. 2B). Further extending the incubation time to 24 h resulted in a greater decrease of dopamine uptake, as both permethrin ($P < 0.01$) and deltamethrin ($P < 0.01$) decreased dopamine uptake by 32 to 42% at 1 μM and 75% at concentrations of 5 and 10 μM (Fig. 2C). To determine the nature of the inhibition of DAT-mediated uptake by deltamethrin and permethrin, we performed kinetic analysis of dopamine uptake in SK-DAT cells exposed to 10 μM of either compound for 24 h. Both pyrethroids showed significant alterations in V_{max}, with variable effects on K_m, suggesting that the decreased uptake may be the result of non-competitive inhibition (Figs. 3A and B). Similar results were observed following 30-min incubations with both compounds (data not shown).

Based upon the time and concentrations required for deltamethrin and permethrin to cause decreased dopamine uptake, we considered that the decreased uptake may be the result of loss of DAT protein. Exposure of cells to 10 μM of deltamethrin or permethrin was without effect on the total levels of DAT as determined by Western immunoblotting (Fig. 3C). We next examined whether exposure to

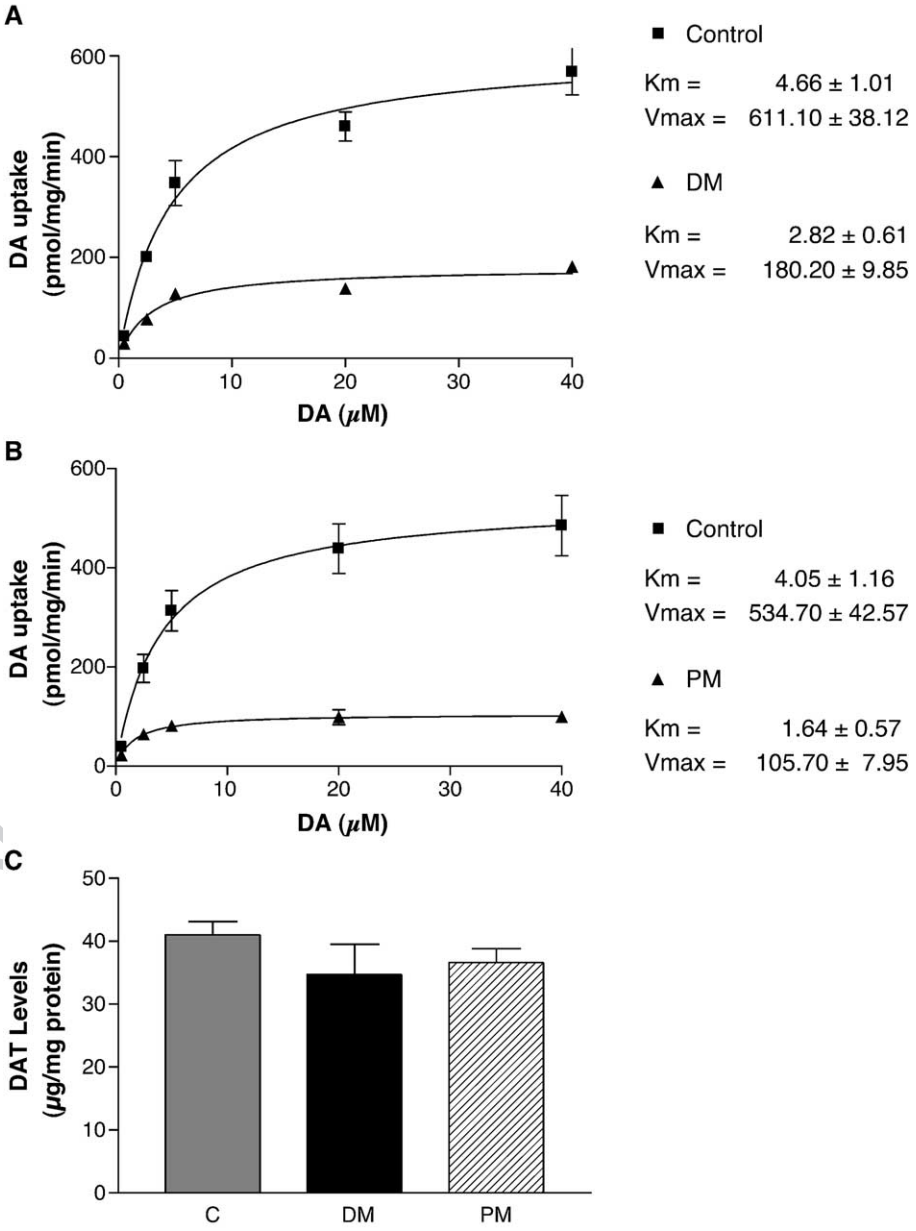

Fig. 3. Effects of (A) deltamethrin (DM; 10 μM) or (B) permethrin (PM; 10 μM) treatment for 24 h on the kinetics of dopamine uptake in SK-N-MC neuroblastoma cells stably expressing the human DAT. Cells were incubated with DM or PM for 24 h and the kinetics of dopamine uptake were determined by using varying concentrations of dopamine as described in Materials and methods. Data represent mean ± SEM ($n = 3$) and absence of error bars indicates that the standard error resides within the size of the symbol. (C) Total DAT levels in cells treated with DM or PM for 24 h as determined by Western immunoblotting. Data represent mean ± SEM ($n = 3$).

deltamethrin or permethrin resulted in cytotoxicity by assessing LDH leakage from the cells into the incubation medium. Treatment of SK-DAT cells with 1 to 10 μM of deltamethrin or permethrin for 24 h did not produce any significant change in LDH leakage (Fig. 4A), effectively ruling out overt cytotoxicity as a mechanism for the decreased dopamine uptake. However, exposure of SK-DAT cells to 5 or 10 μM of permethrin or 10 μM deltamethrin for 24 h significantly increased DNA fragmentation, an indication of an active apoptotic process (Fig. 4B). Exposure to 5 μM permethrin increased the amount of fragmentation by 191% ($P < 0.05$) and exposure to 10 μM increased fragmentation by 422% ($P < 0.01$). Deltamethrin increased fragmentation by 223% ($P < 0.05$) only at 10 μM. Similarly, increased DNA fragmentation was observed following 30 min of exposure to 10 μM of deltamethrin (35%; $P < 0.05$) or permethrin (65%; $P < 0.05$). No significant effects were observed with lower concentrations (data not shown).

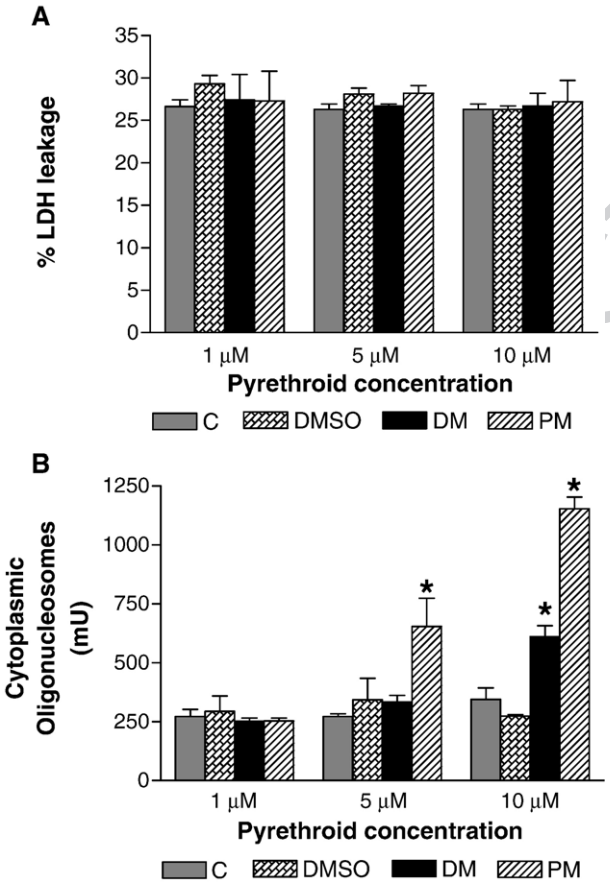

Fig. 4. Effects of deltamethrin or permethrin on (A) LDH leakage and (B) DNA fragmentation. SK-N-MC cells stably expressing the human DAT were treated with media (C), vehicle (DMSO), deltamethrin (DM; 1–10 μM), or permethrin (PM; 1–10 μM) for 24 h. After exposure, cell-free media samples were collected and assayed for LDH levels by spectrophotometry. DNA fragmentation in cells following 24 h of exposure was determined as described in Materials and methods. Data for LDH are presented as percentage of LDH leakage and represent mean ± SEM ($n = 3$). *Groups are significantly different from control values ($P \leq 0.05$) using the untransformed data as determined by ANOVA followed by Dunnett's test.

Discussion

Previous studies have demonstrated that repeated exposure of mice to the pyrethroid pesticides, deltamethrin and permethrin, results in increased synaptosomal dopamine uptake (Kirby et al., 1999; Karen et al., 2001; Gillette and Bloomquist, 2003). In this study, we confirm these observations and extend them by demonstrating that the functional up-regulation is accompanied by increases in DAT-binding sites. In addition, we demonstrate that permethrin and deltamethrin have no direct effect on DAT and that longer-term in vitro exposure of cells stably expressing DAT results in decreased DAT-mediated dopamine uptake and DNA fragmentation.

Deltamethrin and permethrin are members of the pyrethroid class of pesticides which are synthetic derivatives of the naturally occurring pyrethrum from chrysanthemum flowers. These compounds exert their toxicity primarily through binding to sodium channels and prolonging the opening of the channel (Narahashi, 1996; Soderlund et al., 2002). However, recent data suggest that these compounds may specifically target the dopaminergic system. It has been demonstrated that exposure of mice to deltamethrin or permethrin results in an increase in dopamine uptake in striatal synaptosomes, possibly indicative of an up-regulation of DAT (Karen et al., 2001; Kirby et al., 1999). In addition, up-regulation of dopamine uptake following deltamethrin exposure was accompanied by increased binding of ^{3}H-GBR 12935 (Gillette and Bloomquist, 2003). In this study, we found significant increases in DAT-binding sites as measured with ^{3}H-WIN 35,428 that mirrored the increase in DAT-mediated dopamine uptake. While no specific mechanism has been identified for the increase of DAT by these compounds, chemicals known to cause dopamine release, like amantidine and the organochlorine pesticide heptachlor, can increase DAT expression (Gordon et al., 1996; Miller et al., 1999a, 1999b; Page et al., 2000; Kirby et al., 2002). If this were to be sustained over time, one would expect that the elevated extracellular dopamine would increase the expression of the dopamine transporter in an attempt to clear and recycle dopamine. Indeed, deltamethrin has been demonstrated to cause dopamine release from pre-loaded synaptosomes (Kirby et al., 1999; Bloomquist et al., 2002). Another possibility is up-regulation of DAT at the transcriptional level. The transcription factor Nurr1 is critical for the development of the dopaminergic phenotype and has been shown to directly enhance transcription of DAT (Sacchetti et al., 2001; Hermanson et al., 2003). Since Nurr1 transcription is enhanced by neuronal activity and membrane depolarization (Brosenitsch and Katz, 2001), dopamine release and/or blockade of sodium channels by pyrethroids may cause up-

regulation of Nurr1, ultimately leading to increased expression of DAT.

In contrast to the in vivo data, our in vitro results show that short-term (10 min) incubation of SK-DAT cells with deltamethrin or permethrin had no effect on DAT-mediated dopamine uptake. However, if the incubation time was extended to 30 min or 24 h, significant decreases in dopamine uptake were observed. Thus, the reduction of dopamine uptake in SK-DAT cells by deltamethrin and permethrin, is both time and concentration dependent. The lack of a significant effect on dopamine uptake after the 10-min incubation time suggests that both compounds are devoid of any direct effect on dopamine uptake. In addition, the decreases we observed with the longer exposure times were associated with significant changes in V_{max} and little effect on K_m, providing further evidence that pyrethroids do not directly interact with DAT. We also found that these effects were not the result of loss of DAT protein. Since a direct (cocaine-like) action on DAT is excluded by our results, other mechanism(s) appear to be involved in the reduction of dopamine uptake observed here. One possibility is that long-term higher-level exposure to pyrethroids leads to prolonged depolarization (Narahashi, 1982; Tabarean and Narahashi, 2001). Indeed, in vitro exposure of rat striatal synaptosomes to high concentrations (10–50 μM) of veratridine, a sodium channel activator, resulted in decreased dopamine uptake (Holz and Coyle, 1974). It has also been shown that administration. In addition, pyrethroids have been demonstrated to inhibit respiratory chain function in isolated mitochondria at submicromolar levels, which could lead to down-regulation of DAT function (Gassner et al., 1997; Maragos et al., 2002; Braguini et al., 2004). Although there are few studies reporting pyrethroid concentrations in the brain following systemic administration, Sheets (1994) reported that brain levels of deltamethrin were 0.023 μg/g and 0.145 μg/g following a single oral exposure to 4 or 80 mg/kg in adult rats. These brain concentrations are roughly equivalent to 45 and 287 nM, which are in the range of the concentrations used in our in vitro studies that were without effect on the DAT, but lower than those demonstrated to effect mitochondrial function (EC_{50} 793 nM for permethrin and >200 nM for deltamethrin; Gassner et al., 1997; Braguini et al., 2004). Thus, it is likely that the concentrations employed in our in vitro studies are similar to the higher doses of permethrin (25–200 mg/kg) demonstrated to decrease mitochondrial function, dopamine uptake, and DAT immunoreactivity in mice (Karen et al., 2001; Bloomquist et al., 2002; Gillette and Bloomquist, 2003; Pittman et al., 2003).

Since we found that neither deltamethrin nor permethrin has a direct effect on DAT and we estimated that the concentrations employed were similar to those that decrease mitochondrial function, we sought to determine whether the reduction in dopamine uptake was the result of pyrethroid-induced cytotoxicity. Following 30 min or 24 h exposure to 10 μM of deltamethrin or permethrin, there was no significant cytotoxic effect as determined by LDH assay. This is in agreement with the observation that exposure to pyrethroids for 24 h did not produce any significant effect on LDH release from mouse cerebellar granule cells (Imamura et al., 2000), and indicates that the decreased dopamine uptake we observed is not due to cytotoxicity. Since we observed no overt cytotoxicity at any of the concentrations or times tested, we tested the possibility that deltamethrin and permethrin were causing apoptosis. Our findings reveal that both deltamethrin and permethrin induce apoptosis, as indicated by increased DNA fragmentation, following 30-min and 24-h incubations at the highest concentration used (10 μM), whereas at lower concentrations (5 μM), only permethrin induced apoptosis. These data suggest that apoptosis may explain, in part, some of the observed decrease in dopamine uptake in these cells at the higher concentrations used. There is evidence indicating that apoptosis might play a crucial role in the toxic actions of pyrethroids by induction of apoptosis and altering the expression of p53, Bax, and Bcl-2 genes (Wu and Liu, 2000a, 2000b), although the doses used were greater than 4-fold higher than we used in our in vivo studies. Taken together with our results, these studies suggest that higher-level exposure to pyrethroids may result in apoptosis, similar to that seen with our in vitro studies.

The alteration of DAT function and expression by pyrethroid exposure is of particular interest when taken in context of the role of DAT in Parkinson's disease (PD). Several studies have identified pesticide exposure as a risk factor for PD (Priyadarshi et al., 2001). However, the mechanism by which pesticides enhance the risk of PD is not known. Previously, we and others have demonstrated that alterations of DAT expression can greatly affect the vulnerability of the dopamine neuron to neurotoxins such as MPTP or methamphetamine (Gainetdinov et al., 1997, 1998; Donovan et al., 1999; Fumagalli et al., 1998). In addition, the brain regions most vulnerable to parkinsonism-inducing toxin MPTP and those most affected by PD display the highest levels of DAT expression (Miller et al., 1999b; Uhl, 1998). Supporting the observation in humans, animals overexpressing DAT (Donovan et al., 1999) are more susceptible to MPTP toxicity. Therefore, enhanced DAT levels and function by pesticides may increase the susceptibility of dopamine neurons to endogenous neurotoxic dopamine metabolites or exogenous toxicants by increasing uptake through DAT. Additionally, the decreased dopamine uptake and increased DNA fragmentation, suggestive of an ongoing apoptotic process, following in vitro pyrethroid exposure may be relevant to PD as well. Positron emission topographic imaging with ^{11}C-WIN 35,428 has revealed early reductions in DAT levels in mild cases of PD (Frost et al., 1993), suggesting that reductions in DAT levels may be an early indicator of clinical PD in humans. Additionally, activated caspase-3, a primary apoptotic effector, has been demonstrated to precede apoptotic death in human PD brain (Hartmann et al., 2000). Therefore,

lower level exposure to pyrethroids may contribute to PD through up-regulation of DAT and increased uptake of endogenous and exogenous neurotoxicants while increased levels result in apoptotic cell death. However, the exact mechanism of pesticides, including pyrethroids, in the etiology of PD remains to be established.

In conclusion, the present study clearly demonstrates that deltamethrin and permethrin increase DAT and DAT-mediated dopamine uptake in striatal synaptosomes following in vivo exposure. However, in vitro experiments revealed that the in vivo effects are likely indirect as acute in vitro exposure of cells stably expressing DAT had no effect on dopamine uptake. We also found that prolonged higher-level exposure decreases dopamine uptake in SK-DAT cells, which may be due in part to induction of apoptosis. These results may shed light on the mechanisms underlying pyrethroids-induced neurotoxicity and might implicate pyrethroids as environmental risk factors leading to the development of PD.

Uncited references

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Doherty et al., 1988
Forshaw et al., 2000
Grosse et al., 2002
Hudson et al., 1986
Kadous et al., 1994
Thompson et al., 2000
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